ORIGINAL ARTICLE

Major Surgery Suppresses Maximal Production of Helper T-Cell Type 1 Cytokines Without Potentiating the Release of Helper T-Cell Type 2 Cytokines

Ramon Berguer, MD; Capt Normita Bravo, MS, USAF; Mark Bowyer, MD; Craig Egan, MD; Tom Knolmayer, MD; David Ferrick, PhD

Background: Major surgery is known to suppress T-cell function; however, its differential effects on the production of helper T-cell type 1 (T_H1) and type 2 (T_H2) cytokines remains unknown.

Objective: To measure the production patterns of T_H1 (interleukin 2 [IL-2] and interferon γ) and T_H2 (IL-4 and IL-10) cytokines following major surgery.

Design, Setting, and Patients: A cohort study of patients (both active and former members of the armed forces) at a military hospital.

Intervention: Aortic surgery or carotid endarterectomy and measurement of serum IL-6 levels by enzyme-linked immunosorbent assay.

Main Outcome Measures: Unstimulated and stimulated intracellular levels of IL-2, IL-4, IL-10, and interferon γ in $CD4^+$, $CD8^+$, and $\gamma\delta^+$ T cells and serum IL-6 levels immediately before and for 2 days after aortic surgery or carotid endarterectomy.

Results: No unstimulated production of T_H1 or T_H2 cytokines was detected. Stimulated intracellular levels of IL-2 and interferon γ were significantly depressed during the postoperative period in all T-cell subsets in both patient groups. There were no postoperative increases in stimulated IL-4 or IL-10 levels.

Conclusion: Major surgery suppresses the potential responses of T_H1 cytokines without enhancing production of T_H2 cytokines.

Arch Surg. 1999;134:540-544

From the Departments of Surgery (Dr Berguer) and Pathology, Microbiology, and Immunology (Dr Ferrick), University of California, Davis; Clinical Investigation Facility (Capt Bravo) and Surgical Flight (Drs Bowyer, Egan, and Knolmayer), David Grant Medical Center, Travis Air Force Base; Surgical Service, Department of Veterans Affairs, Northern California Health Care System, Martinez (Dr Berguer); and Rigel Inc, Sunnyvale (Dr Ferrick), Calif.

DURING THE past 2 decades, clinicians and researchers have recognized that surgery results in an impaired immune response in the early postoperative period[1] and that this phenomenon may contribute to infectious complications[2] and cancer metastasis.[3] The immunosuppressive effect of surgery has been variously attributed to serum factors,[4] stress hormones,[4,5] the activation of suppressor cells,[5,6] decreased secretion of the cytokines interferon γ (IFN-γ) and interleukin 2 (IL-2),[7,8] and, most recently, to a shift in the balance of helper T-cell type 1 (T_H1) and type 2 (T_H2) cytokines from peripheral blood mononuclear cells.[9,10] A better understanding of perioperative immune function might enable clinicians to stratify patients as immunologically high risk before surgery and take appropriate preventive action or delay surgery. Alternatively, patients demonstrating substantial postoperative immune suppression could be considered for additional prophylactic treatment aimed at reducing the risk of infectious complication.

The present study used recently developed flow cytometry techniques for direct intracellular cytokine measurements along with current concepts of lymphocyte immunomodulation to arrive at a more precise understanding of the effects of surgical injury on immune function. The hypothesis was that surgical trauma suppresses cytokines through an increase in the production of T_H2 cytokines.

RESULTS

The patient characteristics for the 2 surgical groups are listed in the **Table**. Complete blood cell counts (**Figure 1**) demonstrated significant postoperative increases in the numbers of neutrophils in both groups compared with preoperative levels. Absolute lymphocyte counts decreased in both groups compared with preoperative levels. The postoperative platelet count decreased significantly in the aortic surgery group but not in the ca-

PATIENTS AND METHODS

This study consisted of a cohort of 18 patients in the Veterans Affairs and US Department of Defense who underwent elective aortic surgery (n = 10) or carotid endarterectomy (n = 8). The study was approved by the Institutional Review Board at David Grant Medical Center, Travis Air Force Base, Calif, and all patients signed informed consent forms prior to participation in the study. Entry criteria excluded patients with diseases known to affect the immune system, such as acquired immunodeficiency syndrome, chronic hepatitis B and C, known active malignancy, and the use of immunosuppressive medications. Samples of peripheral venous blood (20 mL in EDTA for flow cytometry and 7-mL clot tube for serum assays) were obtained by venipuncture from each patient no more than 6 hours preoperatively and every morning after surgery until discharge from the hospital or for a maximum of 5 days. A group of 17 healthy volunteers were studied as reference subjects.

T-cell subsets and their intracellular cytokine production were identified by multiparametric flow cytometry.[11-13] Peripheral blood mononuclear cells were isolated from 20 mL of anticoagulated (EDTA) venous blood by density centrifugation and incubated at 37°C in a 5% carbon dioxide incubator for 4 hours in RPMI (Roswell Park Memorial Institute; Sigma-Aldrich Corp, St Louis, Mo) medium containing 10-µg/mL brefeldin A (Epicentre Technologies, Madison, Wis) to dissaggregate the Golgi complex and enable the intracellular accumulation of proteins.[14] During the incubation period, half the cells were separately stimulated with 20-ng/mL phorbolmystrateacetate (Sigma-Aldrich Corp) and 1-µg/mL ionomycin (Sigma-Aldrich Corp) to assess submaximal cytokine production, while the other half remained unstimulated. After incubation, cell suspensions were divided into 18 tubes with approximately 1×10^6 cells per tube corresponding to cell type ($CD4^+$, $CD8^+$, or $\gamma\delta^+$), cytokine combination (isotype control, IFN-γ/IL-4, or IL-2/IL-10), and stimulation category (unstimulated or stimulated). Cells were resuspended in buffer (phosphate-buffered saline plus 0.1% sodium azide plus 1% bovine serum albumin plus 5-mmol EDTA) and incubated for 15 minutes at 25°C with a 1:5 dilution of Tricolor-conjugated (a tandem conjugate of phycoerythrin and Cy-5; Caltag Laboratories, San Francisco, Calif) anti-γδ T-cell receptor, anti-CD4, or anti-CD8 monoclonal antibodies for phenotypic identification. After washing in phosphate-buffered saline, cells were fixed in 100 µL of solution A (cell permeabilization fixation; Caltag Laboratories) for 15 minutes at 25°C and washed again in phosphate-buffered saline. Intracellular cytokines were identified by resuspension in 50 µL of the permeabilization solution B (Caltag Laboratories) containing a 1:500 dilution of dinitrophenyl-conjugated anti–IFN-γ and a 1:100 dilution of biotin-conjugated anti–IL-4; or dinitrophenyl-conjugated anti–IL-10 and biotin-conjugated anti–IL-2; or dinitrophenyl-conjugated anti-mouse IgG1 and biotin-conjugated anti-mouse IgG2 for 15 minutes at 25°C. Specific intracellular cytokine identification was achieved by subsequent incubation (Strepavidin Red 670; Pharmingen Inc, San Diego, Calif) and fluorescein isothiocyanate–conjugated anti-dinitrophenyl antibody (Molecular Probes Inc, Eugene, Ore) for 15 minutes at 25°C. Finally, cells were resuspended in 0.5 mL of cytoflow buffer for analysis. Surface and intracellular fluorescence was measured using a flow cytometer (FACScan; Becton Dickinson Immunocytometry Systems, San Jose, Calif) and fluorescence data were analyzed with software (CellQuest; Becton Dickinson Immunocytometry Systems). After standard calibration of the flow cytometer, data were recorded until 5000 cells were acquired or the cell suspension volume of 0.5 mL was exhausted. The minimum number of cells was lowered to 2000 cells for $\gamma\delta^+$ T cells since these cells are much scarcer in the peripheral blood. Intracellular cytokine detection was performed by double gating each cell surface marker and a lymphocyte gate and measuring fluorescence channel 1 (fluorescein isothiocyanate) and fluorescence channel 3 (Strepavidin Red 670) fluorescence intensity. The data recorded included the total number of acquired cells, the percentage of total cells gated for each subset, and the number of cells positive for fluorescence channel 1 and fluorescence channel 3 internal fluorescence. Background fluorescence was measured using isotype-matched fluorochrome-conjugated IgG and arithmetically subtracted from the measured intracellular cytokine fluorescence to obtain the net detectable intracellular fluorescence. The average background fluorescence measured was less than 1%. Reference assays using fixed, but not permeabilized, cells demonstrated a reduction in the intracellular detection of IL-2 and IFN-γ by 99% and 98%, respectively. Preincubation with recombinant human cytokines before intracellular staining reduced intracellular detection of IL-2 and IFN-γ by 99% and 98%, respectively. Serum IL-6 levels were measured by enzyme-linked immunosorbent assay by the Clinical Investigation Facility at David Grant Medical Center.

A complete data set in both surgical groups was obtained for the preoperative point and the first 2 postoperative days. These data were analyzed using 2-way repeated-measures analysis of variance and Tukey post hoc test. Patient characteristics were compared using χ^2 analysis for proportional data and the Kolmogorov-Smirnov test for unpaired comparisons that were not normally distributed. All statistical analyses were performed with statistical software (STATISTICA; StatSoft, Tulsa, Okla) for Windows (version 5.0; Microsoft Corporation, Redmond, Wash).

rotid surgery group. Postoperative serum IL-6 levels were significantly elevated in the aortic surgery group only (Figure 1).

Unstimulated intracellular cytokine production was not detectable above nonspecific background activity in any T-cell subset. The stimulated production of the intracellular T_H1 cytokines IFN-γ and IL-2 was significantly decreased in the postoperative period in all T-cell subsets and in both surgical groups compared with preoperative values (**Figure 2** through **Figure 4**, C and D). There were no increases in T_H2 cytokine production after surgery. Small preoperative differences (<1.5%) in the stimulated production of IL-4 in $CD4^+$ and $CD8^+$ cells and IL-10 in γδ T cells between surgery groups were at the threshold of nonspecific background activity, although the differences were statistically significant (Figures 2-4, A and B). There were no significant differences in preoperative or postoperative stimulated

Patient Characteristics for the Surgical Groups*

Characteristic	Aortic Surgery† (n = 10)	Carotid Endarterectomy (n = 8)	P
Mean age, y	67.7	68.3	NS
Male/female	9/1	8/0	NS
Diabetes	10	13	NS
Coronary artery disease	50	25	NS
Smoker	60	38	NS
NSAID user	40	63	NS
Autologous donation	90	0	.002
Autologous transfusion	90	0	.002
Homologous transfusion	20	0	NS
Mean blood loss, mL	1685	147	<.001‡
Infectious complications	30	25	NS
Mean length of stay, d	8.6	5.4	<.005‡

*Values are expressed as percentages except where noted. NS indicates not significant (level of significance at P<.05); NSAID indicates nonsteroidal anti-inflammatory drug.

†Seven patients underwent abdominal aortic aneurysmal repair and 3, aortic bifemoral grafting.

‡Kolmogorov-Smirnov test.

intracellular cytokine production between patients when grouped by operation type, preoperative cardiovascular risk factors (diabetes mellitus, history of coronary artery disease, and smoking), the presence of homologous or autologous transfusion, intraoperative blood loss, operating time, hospital stay, or the development of infectious complications.

COMMENT

There were no significant postoperative changes in unstimulated production of T_H1 or T_H2 cytokines after major vascular operations. These results suggest that major surgery does not trigger a systemic antigen-specific immune response. In contrast, stimulated (potential) production of T_H1 cytokines in $CD4^+$, $CD8^+$, and $\gamma\delta^+$ T cells significantly decreased in the postoperative period ($P<.05$). This effect persisted through the second postoperative day and was independent of the level of the type of operation performed. The decrease in stimulated production of T_H1 cytokines was not accompanied by a simultaneous postoperative increase in production of T_H2 cytokines. There were no differences in the cytokine responses when patients were stratified by preoperative medical diagnosis or by the presence of postoperative complications, although the small sample size limits the power of this observation.

The results of the present study are consistent with previous reports[15-20] that demonstrated that surgical trauma results in a depression of in vitro lymphocyte blastogenesis and proliferation during the early postoperative period. This effect has been attributed to stress-mediated decreases in the production of the T_H cytokines IL-2 and IFN-γ[8,21-23] when measured in bulk supernatants from in vitro cultures of peripheral blood mononuclear cells. More recently, immunosuppression in human disease has been attributed to a shift in cytokine production from a T_H1 (IL-2 and IFN-γ) to a T_H2 (IL-4 and IL-10) pattern.[24] The present study confirms a specific negative effect of surgical injury on the production of T_H1 cytokines without a simultaneous increase in the production of T_H2 cytokines. These findings differ from recent reports by Decker et al[9,10] that suggested that postoperative suppression of T_H1 cytokine responses is associated with an increase in T_H2 cytokine production.

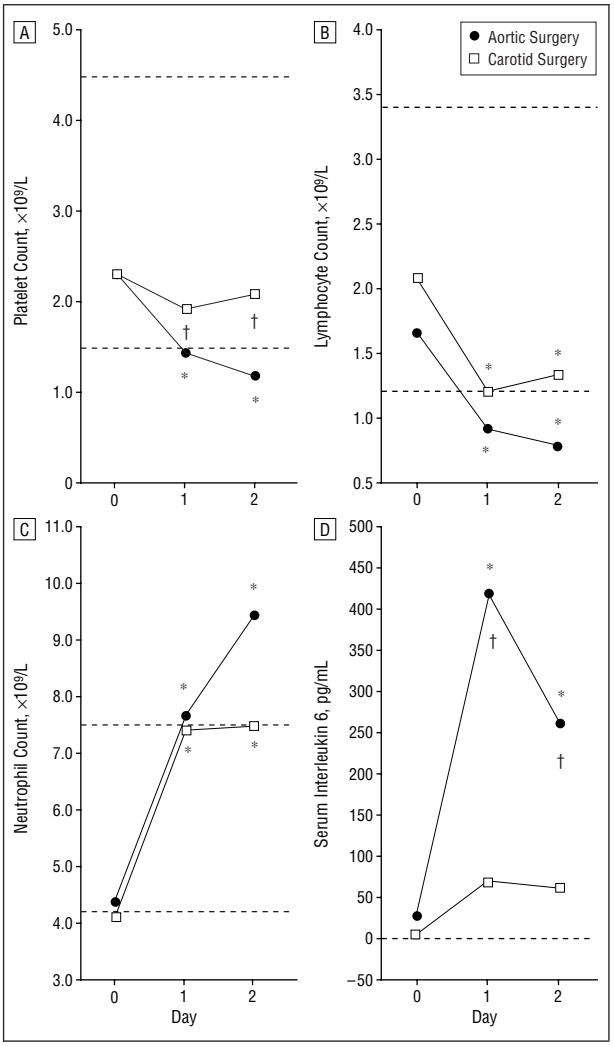

Figure 1. *Results of complete blood cell count and serum interleukin 6 measurements in 18 patients undergoing vascular surgery. Dashed lines represent the upper and lower 95% confidence intervals obtained from 17 reference subjects. Statistical analysis performed using analysis of variance and Tukey post hoc test. The asterisk indicates P<.05 vs preoperative levels; dagger, P<.05 aortic vs carotid surgery groups.*

One explanation for the lack of an observed T_H2 cytokine response in the present study may be a high threshold of detection resulting from the use of a 2-step staining technique that might mask the expected 1% to 3% of T cells that produce IL-4 in response to phorbolmystrateacetate and ionomycin stimulation.[11] The determination of the exact patterns of postoperative cytokine production is also difficult because of the uncertainty of the precise timing of potential T_H2 responses after injury. The results of animal studies demonstrated that the T_H2 cytokine IL-4 is detectable in splenocytes within 24 hours of traumatic injury,[25] whereas the same cytokine is not measurable in splenic and peritoneal cavity T cells until 5 to 8 days after intraperitoneal infection with *Nippostrongylus brasiliensis*, a potent inducer of T_H2.[14] Therefore, it is possible that the first cytokine measurement at

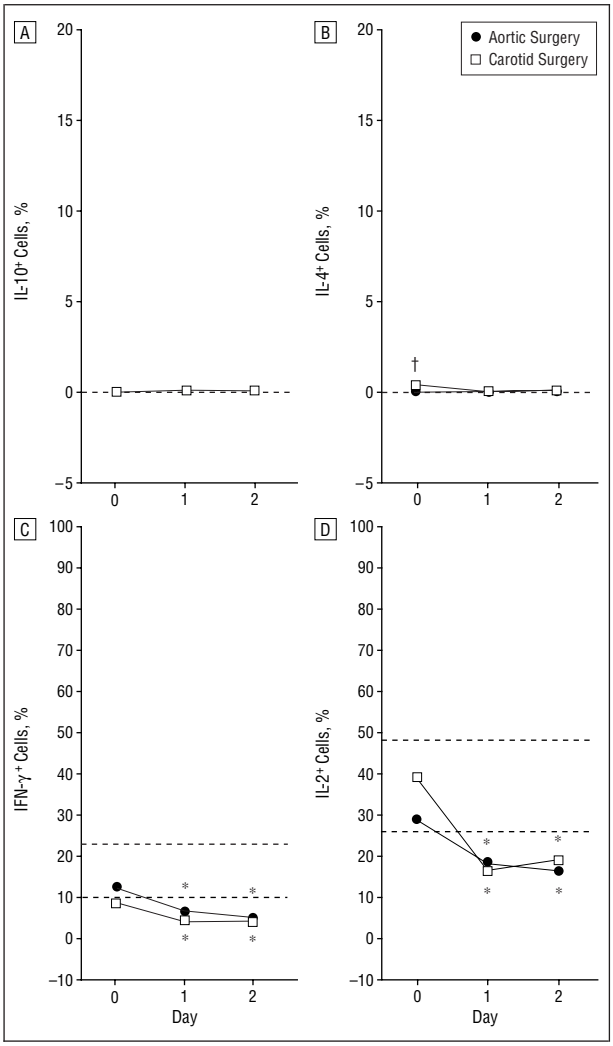

Figure 2. *Results of intracellular helper T-cell type 1 (T_H1) and type 2 (T_H2) cytokine measurements in CD4+ lymphocytes using multiparameter flow cytometry in 18 patients undergoing vascular surgery. IL indicates interleukin; plus sign, postive; and IFN, interferon. See legend for Figure 1 for definition of dashed lines, statistical analyses, asterisk, and dagger.*

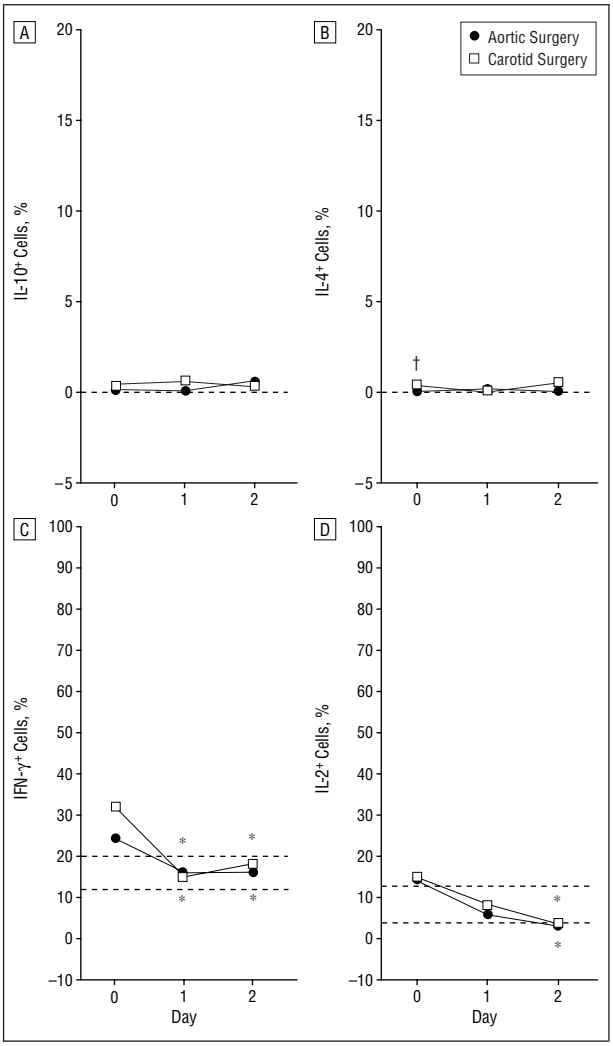

Figure 3. *Results of intracellular helper T-cell type 1 (T_H1) and type 2 (T_H2) cytokine measurements in CD8+ lymphocytes using multiparameter flow cytometry in 18 patients undergoing vascular surgery. IL indicates interleukin; plus sign, positive; and IFN, interferon. See legend for Figure 1 for definition of dashed lines, statistical analyses, asterisk, and dagger.*

24 hours postoperatively was too late to detect an early T_H2 response, which was reported by others.[10] It is also plausible that T_H2 responses developed in patients after the last measurement on postoperative day 2. However, a review of the limited data obtained in the present study between postoperative days 2 and 5 demonstrated no T_H2 cytokine production. Finally, cytokine production in the present study was only measured in T cells, not in other antigen-presenting cells or natural killer cells. These other cell types have been reported to produce T_H2 cytokines[24] in humans. Thus, T_H2 cytokine production by cells other than T cells may explain other reports[10] of postoperative elevations of T_H2 cytokine levels not seen in the present study. One study[24] suggested, however, that it would be unlikely to observe T_H2 cytokine responses in humans that do not involve $CD4^+$ T cells.

The observation that the degree of postoperative suppression of T_H1 cytokine responses was the same after carotid endarterectomy and aortic surgery was surprising. This finding suggests the occurrence of a generalized suppression of T-cell function during the postoperative period that may be independent of the degree of tissue injury. Such a generalized immune suppression following surgical injury may change the immunological significance of what has been defined as major and minor surgery. An unexpected finding was the large variance in preoperative stimulated intracellular cytokine levels in the aortic surgery group; this variance was not present in the carotid endarterectomy group. The difference in preoperative immune reactivity between groups may reflect differences in the patient population, underlying vascular disease, or other unmeasured confounding factors. The potential correlation of decreased preoperative immune function in patients undergoing aortic surgery needs to be further investigated with respect to the risk of postoperative infectious complications.

The initial cytokine milieu is one of the main factors determining T_H1 and T_H2 differentiation in T cells. γδ T cells are an epithelial-associated lymphocyte subset that respond early to infection and cell injury.[26,27] These specialized lymphocytes can release T_H1 and T_H2 cytokines, which are thought to play a role in modulating the

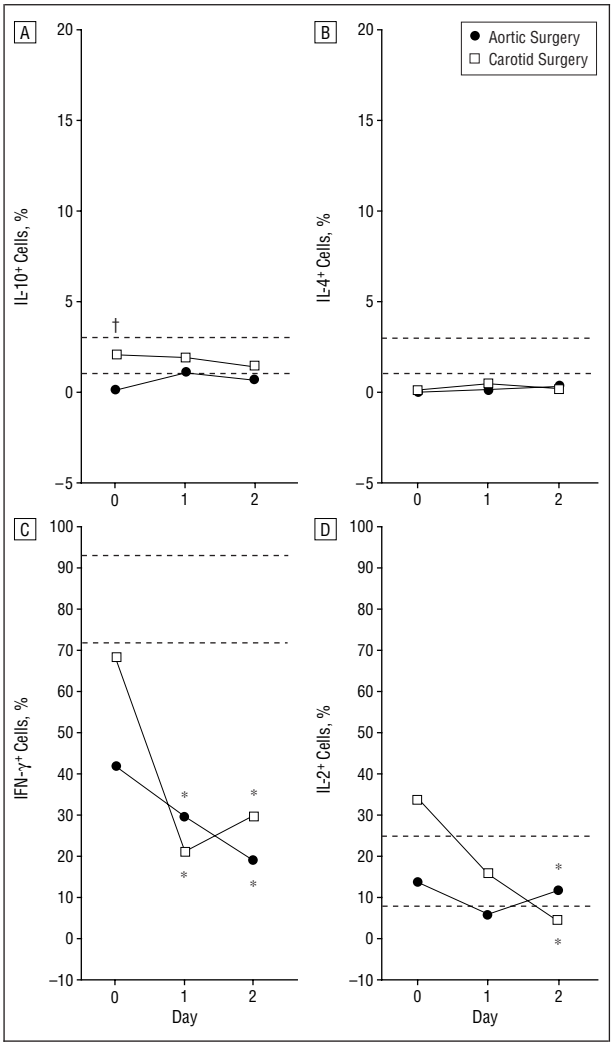

Figure 4. *Results of intracellular helper T-cell type 1 (T_H1) and type 2 (T_H2) cytokine measurements in T-cell receptor γδ+ lymphocytes using multiparameter flow cytometry in 18 patients undergoing vascular surgery. IL indicates interleukin; plus sign, positive; and IFN, interferon. See legend for Figure 1 for definition of dashed lines, statistical analyses, asterisk, and dagger.*

functional differentiation of the T-cell response.[14] The present study demonstrates that γδ T cells are vigorous producers of IFN-γ, a finding reported by other investigators.[14,28,29] The finding that the responses of γδ T-cell T_H1 cytokines are significantly down-regulated after surgery merits further investigation in patients who have undergone surgery because γδ T cells play an important role in the epithelial barrier to bacterial infections,[30] in neutrophil recruitment[26,31] and the process of epithelial repair.[32]

In conclusion, the present study demonstrates that surgical trauma suppresses T_H1 cytokine responses without increasing T_H2 cytokine production. These results provide further information that may direct future treatments based on the T_H1 and T_H2 paradigm focused on decreasing the risk of postoperative infectious complications.

Reprints: Ramon Berguer, MD, Surgical Service, Department of Veterans Affairs Northern California Health Care System, 150 Muir Rd, Mail Code 112, Martinez, CA 94553 (e-mail: berguer.ramon@martinez.va.gov).

REFERENCES

1. Walton B. Anaesthesia, surgery and immunology. *Anaesthesia.* 1978;33:322-348.
2. Christou NV, Meakins JL, Gordon J, et al. The delayed hypersensitivity response and host resistance in surgical patients: 20 years later. *Ann Surg.* 1995;222:534-546.
3. Lundy J, Lovett EJ, Wolinsky SM, Conran P. Immune impairment and metastatic tumor growth. *Cancer.* 1979;43:945-951.
4. Lundy J, Ford CM. Surgery, trauma and immune suppression: evolving the mechanism. *Ann Surg.* 1983;197:434-438.
5. Berkenbosch F, Wolvers DA, Derijk R. Neuroendocrine and immunological mechanisms in stress-induced immunomodulation. *J Steroid Biochem Mol Biol.* 1991; 40:639-647.
6. Hansbrough JF, Bender EM, Zapata-Sirvent R, Anderson J. Altered helper and suppressor lymphocyte populations in surgical patients: a measure of postoperative immunosuppression. *Am J Surg.* 1984;148:303-307.
7. Abe Y, Miyake M, Horiuche A, Kumori K, Kimura S. Changes in the productivity of cytokines and active-oxygen in peripheral blood cells following surgery. *Surg Today.* 1992;22:15-18.
8. Akiyoshi T, Koba F, Arinaga S, Miyazaki S, Wada T, Tsuji H. Impaired production of interleukin-2 after surgery. *Clin Exp Immunol.* 1985;59:45-49.
9. Decker D, Lindemann C, Decker P, Kania U, Hirner A, von Rucker A. Effect of endoscopic pre-peritoneal inguinal hernia repair and Shouldice inguinal hernia repair on T-cell differentiation [in German]. *Langenbecks Arch Chir Suppl Kongressbd.* 1997;114:261-265.
10. Decker D, Schondorf M, Bidlingmaier F, Hirner A, von Ruecker AA. Surgical stress induces a shift in the type-1/type-2 T-helper cell balance, suggesting down-regulation of cell-mediated and up-regulation of antibody-mediated immunity commensurate to the trauma. *Surgery.* 1996;119:316-325.
11. Jung T, Schauer U, Heusser C, Neumann C, Rieger C. Detection of intracellular cytokines by flow cytometry. *J Immunol Meth.* 1993;159:197-207.
12. Crucian B, Dunne P, Friedman H, Ragsdale R, Pross S, Widen R. Detection of altered T helper 1 and T helper 2 cytokine production by peripheral blood mononuclear cells in patients with multiple sclerosis utilizing intracellular cytokine detection by flow cytometry and surface marker analysis. *Clin Diagn Lab Immunol.* 1996;3:411-416.
13. Openshaw P, Murphy EE, Hosken NA, et al. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *J Exp Med.* 1995;182:1357-1367.
14. Ferrick DA, Schrenzel MD, Mulvania T, Hsieh B, Ferlin WG, Lepper H. Differential production of interferon-gamma and interleukin-4 in response to T_H1- and T_H2-stimulating pathogens by gamma delta T cells in vivo. *Nature.* 1995;373:255-257.
15. Berenbaum MC, Fluck PA, Hurst NP. Depression of lymphocyte responses after surgical trauma. *Br J Exp Pathol.* 1973;54:597-607.
16. Cullen BF, Belle GV. Lymphocyte transformation and changes in leukocyte count: effects of anesthesia and operation. *Anesthesiology.* 1975;43:563-569.
17. Vose BM, Moudgil GC. Post-operative depression of antiboty-dependent lymphocyte cytotoxicity following minor surgery and anaesthesia. *Immunology.* 1976; 30:123-128.
18. Park SK, Brody JI, Wallace HA, Blakemore WS. Immunosuppressive effect of surgery. *Lancet.* 1971;1:53-55.
19. Lennard JWJ, Shenton BK, Borzotta A, et al. The influence of surgical operations on components of the human immune system. *Br J Surg.* 1985;72:771-776.
20. Salo M. Effects of anesthesia and surgery on the immune response. *Acta Anesthesiol Scand.* 1992;36:201-220.
21. Hensler T, Hecker H, Heeg K, et al. Distinct mechanisms of immunosuppression as a consequence of major surgery. *Infect Immun.* 1997;65:2283-2291.
22. Deehan DJ, Heys SD, Simpson W, Broom J, McMillan DN, Eremin O. Modulation of the cytokine and acute-phase response to major surgery by recombinant interleukin-2. *Br J Surg.* 1995;82:86-90.
23. Chu SH, Hu RH, Lee YC, Chen KT. Changes of white blood cells, immunosuppressive acidic protein, and interleukin-2 receptor after open heart surgery. *Thorac Cardiovasc Surg.* 1995;43:94-98.
24. Lucey DR, Clerici M, Shearer GM. Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic, and inflammatory diseases. *Clin Microbiol Rev.* 1996; 9:532-562.
25. Napolitano L, Campbell C, Bass B. Kinetics of splenocyte interleukin-4 production after injury and lethal endotoxin challenge. *J Surg Res.* 1997;67:33-39.
26. Ferrick D, Braun R, Lepper H, Schrenzel M. Gamma-delta T-cells in bacterial infections. *Res Immunol.* 1996;147:532-541.
27. Groh V, Steinle A, Bauer S, Spies T. Recognition of stress-induced MHC molecules by intestinal epithelial gamma delta T cells. *Science.* 1998;279:1737-1740.
28. Simpson SJ, Hollander GA, Mizoguchi E, et al. Expression of pro-inflammatory cytokines by TCR alpha beta+ and TCR gamma delta+ T cells in an experimental model of colitis. *Eur J Immunol.* 1997;27:17-25.
29. Berguer R, Ferrick DA. Differential production of intracellular γ-interferon in αβ and γδ T-cell subpopulations in response to peritonitis. *Infect Immun.* 1995;63:4957-4958.
30. Cheng SH, Cheng SH, Penninger JM, et al. Biology of murine gamma delta T cells. *Crit Rev Immunol.* 1991;11:145-166.
31. Boismenu R, Feng L, Xia YY, Chang JC, Havran WL. Chemokine expression by intraepithelial gamma delta T cells: implications for the recruitment of inflammatory cells to damaged epithelia. *J Immunol.* 1996;157:985-992.
32. Boismenu R, Havran WL. Modulation of epithelial cell growth by intraepithelial gamma delta T cells. *Science.* 1994;266:1253-1255.